EXHIBIT X

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Immunology

Monoclonal antibodies specific for carcinoembryonic antigen and produced by two hybrid cell lines

(tumor antigen/hybridoma/serology)

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ABSTRACT **Spleen cells from mice immunized with purified carcinoembryonic antigen (CEA), an important tumor marker of human carcinomas, were fused with the mouse myeloma cell line P3-NSI/1-Ag4. Out of the 400 hybrids obtained, 2 secreted antibodies reacting specifically with two different antigenic determinants present on CEA molecules. They were cloned and established as permanent hybridoma cell lines. These antibodies, which have relatively high affinities and can be produced in unlimited amounts, will be useful both for the immunochemical characterization of CEA and as a standard reagent for the identification of this antigen in human tissues and body fluids.**

Carcinoembryonic antigen (CEA) is a glycoprotein of 180,000 daltons described in 1965 by Gold and Freedman (1) as an antigen present exclusively in adenocarcinoma of the human digestive tract and in digestive organs from fetuses of 2- to 6-month gestation. The tumor and organ specificity of CEA, however, was challenged by several reports describing small amounts of substances immunologically identical to CEA in normal colon mucosa (2–4) and in carcinomas from nondigestive organs (5). In addition, it has been shown that CEA shares antigenic determinants with crossreacting substances (6–8) present in large amounts in normal adult tissues. In the light of these findings, it appears important to obtain monoclonal antibodies that recognize only CEA-specific antigenic determinants. Using the method of Köhler and Milstein (9), we fused spleen cells from CEA-immunized mice with a mouse myeloma cell line. Although CEA is a good immunogen in the mouse, for unknown reasons, we, like other groups, had a long series of negative results. Recently, however, we obtained two hybrids (VII-23 and VII-37) producing antibodies reacting specifically with CEA. The properties of these two monoclonal antibodies against CEA will be described here.

MATERIALS AND METHODS

Immunization and Fusion Protocol. BALB/c mice 3–4 months old were immunized by two injections of 15 μg of CEA purified from colon carcinoma as described (5). The first injection was given intraperitoneally with complete Freund's adjuvant, the second intravenously in saline 2 months later. After 3 days from boost mouse spleen was aseptically removed and fusion was performed by incubating 10^8 spleen cells with 10^7 P3-NSI/1-Ag4 myeloma cells (10) in 0.3 ml of 40% (vol/vol) polyethylene glycol (11, 12) M_r 1000 (Merk, Darmstadt, West Germany) for 3 min at 37 °C. The cells were centrifuged for 5 min at 200 × *g*, then 5 ml of serum-free Dulbecco's modified Eagle's medium was added dropwise to dilute the polyethylene glycol. After fusion, the cells were washed and resuspended in 100 ml of Dulbecco's modified Eagle's medium containing hypoxanthine, aminopterin, and thymidine (13), 10% gamma globulin-free horse serum (GIBCO), gentamicin (20 μg/ml), and 2 mM glutamine (HAT medium). They were then distributed in 96 wells 2 cm in diameter (Nunc, Denmark). After 2 weeks of culture, growing hybrids were found in almost all wells.

Antibody Detection Assay. Culture fluids of growing hybrids were tested for the presence of anti-CEA antibody by a radioimmunoassay using ^{125}I-labeled purified CEA from a preparation different from the one used for immunization. Briefly, 200 μl of culture fluid was incubated for 2 hr at 37°C with 0.2 ng of ^{125}I-labeled CEA. CEA bound to antibodies was precipitated at 4°C by adding ammonium sulfate to 50% saturation in the presence of normal human serum diluted 1:100.

Isotype Assay. The isotype of the positive hybrid culture fluids was determined by using a solid-phase radioimmunoassay (14, 15) in which unlabeled CEA at a concentration of 0.1 mg/ml in phosphate-buffered saline was adsorbed to wells of polyvinyl plates, 0.1 ml per well and then incubated sequentially with 0.1 ml of culture fluids, appropriate dilution in phosphate-buffered saline of goat antiserum specific for mouse IgM, IgG_1, IgG_2, or IgA (Meloy, Springfield, VA) and finally with purified ^{125}I-labeled rabbit antibodies against goat IgG. All incubation steps, lasting 2 hr each, were done at room temperature.

Internal Labeling of Hybrid Products. Antibodies from positive hybrids were internally labeled by addition of 10 μCi (1 Ci = 3.7 × 10^{10} becquerels) of [^{3}H]leucine to cultures of 10^6 hybrid cells in 1 ml of leucine-free HAT medium. After 16-hr incubation at 37°C, culture fluids were harvested and tested immediately or kept frozen at −20°C.

Determination of Affinity Constant (K_a) of Monoclonal Antibodies. To a limited amount of monoclonal antibody, increasing amounts of ^{125}I-labeled CEA were added in a final volume of 350 μl of 0.02 M Tris-HCl buffer, pH 7.4. After 16-hr incubation at 37 °C, CEA bound to antibodies was precipitated at 4 °C by adding ammonium sulfate to 50% saturation in the presence of normal human serum diluted 1:100. To calculate K_a, saturation curves obtained at equilibrium were transformed by double reciprocal plot of 1/antigen bound as a function of 1/antigen free as described (14, 16).

RESULTS AND DISCUSSION

More than 400 hybrids deriving from seven different fusions were screened for anti-CEA activity. Culture fluids from nine hybrids showed some CEA binding activity, but only two of them (VII-23 and VII-37) remained positive after subculture

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Abbreviations: CEA, carcinoembryonic antigen; HAT medium, hypoxanthine aminopterin thymidine medium; NCP, normal glycoprotein crossreacting with CEA

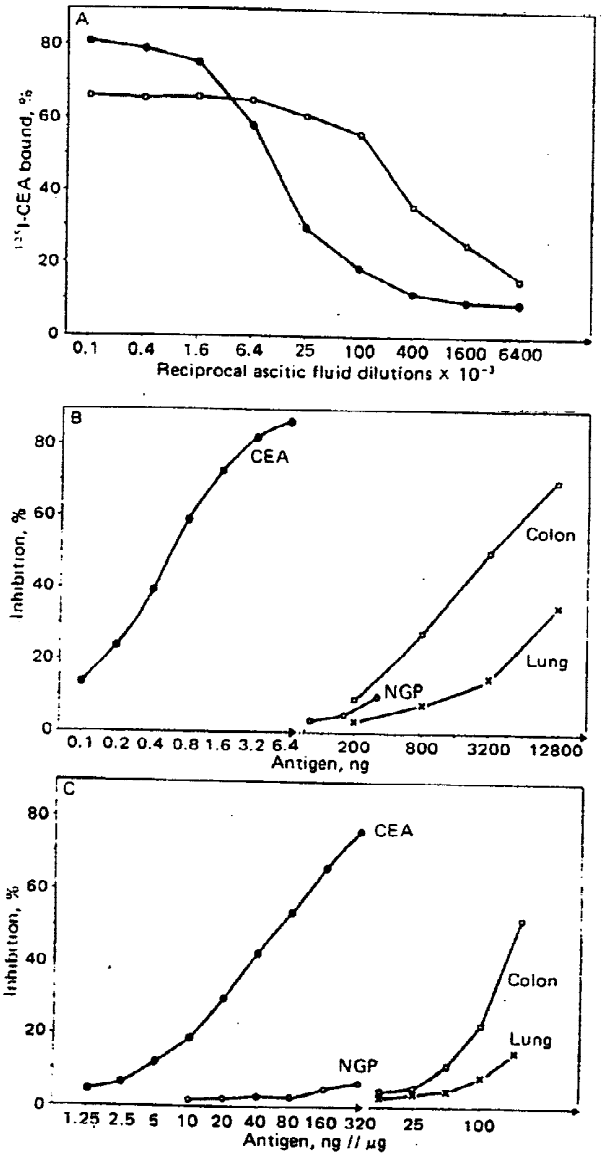

FIG. 1. (*A*) Titration curves of ascitic fluids produced by the anti-CEA clones VII-23e (●) and VII-37a (□). Two hundred µl of 1:4 dilutions of ascitic fluids were incubated for 2 hr at 37°C with 0.2 ng of ^{125}I-labeled CEA. CEA bound to antibodies was precipitated at 4°C by adding ammonium sulfate to 50% saturation in the presence of normal human serum diluted 1:100. The background CEA precipitation in absence of ascitic fluid was 8%. (*B*) Inhibition of binding of labeled CEA with a constant amount of clone VII-23e antibodies (ascitic fluid diluted 1:15,000) by increasing concentrations of purified CEA (●), the purified crossreacting antigen NGP (○), or crude perchloric acid extracts of normal colonic mucosa (□) or normal lung (×). Note that there is an interruption of the abscissa scale between 6.4 and 100 ng. (*C*) Inhibition of binding of labeled CEA with a constant amount of clone VII-37a antibodies (ascitic fluid diluted 1:400,000) by the same substances as in *B*. Note that there is an interruption of the abscissa scale between 320 ng and 12,500 ng (12.5 µg). All the radioimmunoassays in *A*, *B*, and *C* were performed in 0.02 M Tris-HCl buffer, pH 7.4.

of the hybrid cells. The cells from these two hybrids were cloned in a limiting dilution system in 96-well plates in the presence of normal syngeneic macrophages as a feeding layer. Anti-CEA antibodies produced by clone VII-23e derived from hybrid 23

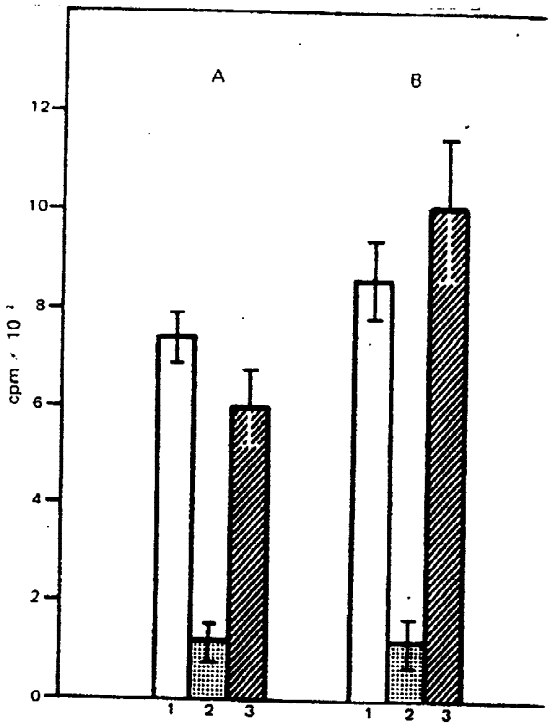

FIG. 2. Competition of binding to insolubilized CEA betwe antibodies from clones VII-23e and VII-37a. Antibodies from positi clones were internally labeled with [^{3}H]leucine. A 25-µl sample of e labeled culture fluid was incubated for 2 hr with CEA adsorbed to wells of a polyvinyl plate (14). For competition analysis, 25 µl of labeled culture fluid from a 3-day culture of each clone was allow to react for 10 min with the adsorbed CEA before the addition of µl of labeled culture fluid from each clone. After 2 hr, the wells w washed, and the radioactivity bound to them was measured in a liq scintillation counter. The solid lines represent the standard deviati from three separate experiments. (*A*) Labeled culture fluid fr VII-23e: 1, without unlabeled culture fluid; 2, after addition of un beled culture fluid from VII-23e; 3, after addition of unlabeled cult fluid from VII-37a. (*B*) Labeled culture fluid from VII-37a: 1, with unlabeled culture fluid; 2, after addition of unlabeled culture fl from VII-37a; and 3, after addition of unlabeled culture fluid fr VII-23e.

and clone VII-37a derived from hybrid 37 were further an lyzed. For simplicity we will refer to them as 23 and 37.

Isotype of the Two Monoclonal Antibodies. Table 1 sho results of the isotype analysis of the antibodies produced by t two clones. The results indicate that antibodies from clone are of the IgG_1 subclass, whereas those from clone 37 are of t IgG_2 subclass.

Titration of Hybridoma Products. The cells from bo clones were injected intraperitoneally into BALB/c mice, whe they produced ascitic tumors secreting large amounts of an CEA antibodies. Representative titration curves of ascitic flu produced by the two clones are shown in Fig. 1*A*. Ascitic flu from clone 23 bound up to 80% of purified labeled CEA but ha a lower titer than ascitic fluid from clone 37, which bound on 65% of labeled CEA.

Specificity of the Two Hybridoma Antibodies. The CE specificity of the antibodies from the two clones was demon strated by inhibition curves obtained with increasing concen trations of unlabeled CEA in comparison with purified CEA crossreacting antigen (NGP) (6, 17) or crude 0.6 M perchlor acid extracts of normal colonic mucosa and normal lung. Fig 1*B* shows that, in the radioimmunoassay developed with clon

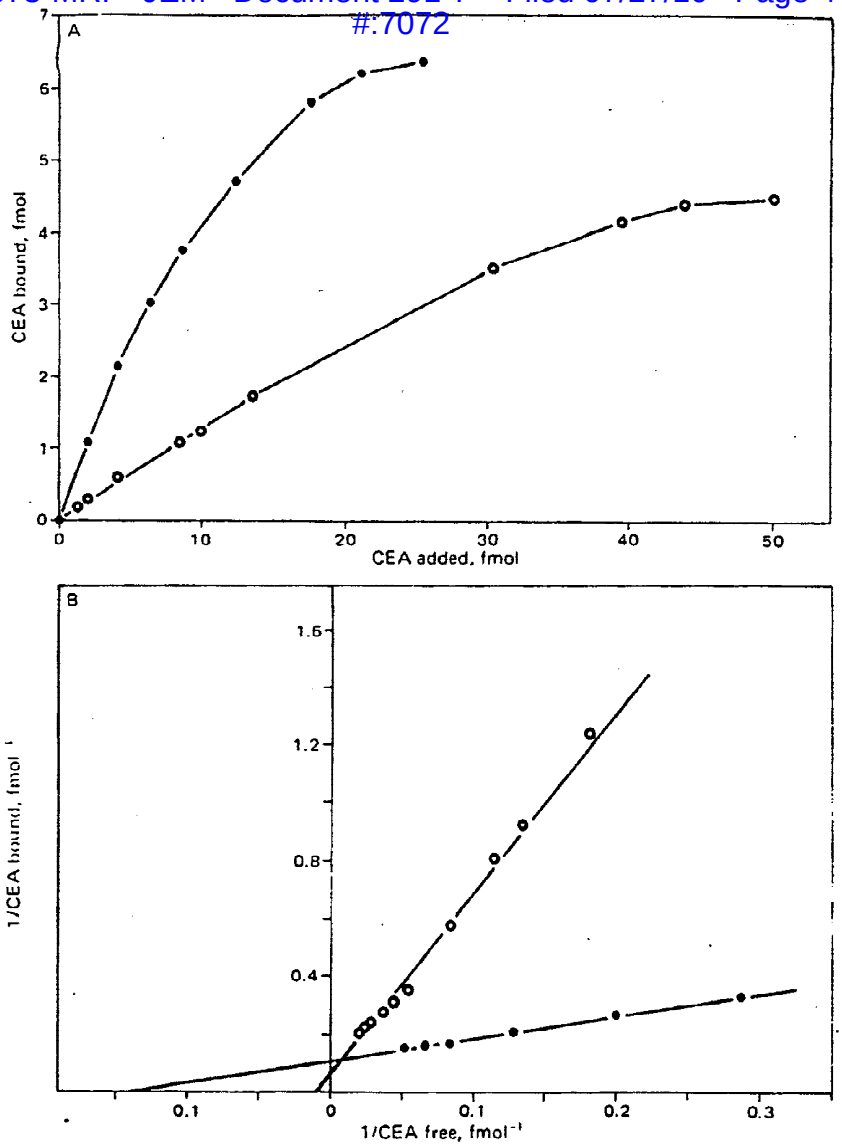

FIG. 3. (*A*) Saturation curve of anti-CEA antibodies from clones VII-23e (●) and VII-37a (○). The test was carried out by using ascitic fluids diluted 1:6000 for VII-23e and 1:100,000 for VII-37a. All determinations were done in duplicate. For each CEA concentration the background of CEA precipitation in the absence of antibodies was determined (it ranged from 7% to 9%) and was subtracted from the values obtained with antibodies. (*B*) Double reciprocal plot of the data shown in *A*. Concentrations refer to CEA at equilibrium. The intercept on the ordinate indicates the maximum binding capacity of the sample at saturation (i.e., in infinite excess of antigen). The intercept on the negative extension of the abscissa represents the affinity expressed as K_a and corresponds to the concentration of free antigen at which the binding reached 50% of the maximum value. Note that the first two points of the saturation curve of VII-23e are not included in the corresponding reciprocal plot because they fell far out of the abscissa scale. In an extended scale they fitted exactly with the extension of the line shown.

23 antibodies, 50% inhibition was obtained with 0.4 ng of purified CEA, whereas 320 ng of NGP was required to obtain 10% inhibition. Furthermore, 3200 ng of crude perchloric acid extracts of normal colon mucosa and normal lung were necessary to give 50% and 15% inhibition, respectively. The inhibitions observed with a large excess of crude perchloric acid extracts might be due to the small amount of CEA present in normal colon and normal lung. It is important to mention that CEA preparations purified from normal colon mucosa (3) were able to inhibit the radioimmunoassay developed with the two monoclonal antibodies almost as efficiently as CEA purified from colon carcinoma (data not shown). Fig. 1C shows that the radioimmunoassay developed with clone 37 antibodies gave a similar degree of specificity for CEA but 1/70th the sensitivity of the assay using antibodies from clone 23. Furthermore, we recently demonstrated (18) by the indirect immunoperoxidase method that the two monoclonal antibodies reacted specifically with frozen sections or cell lines from colon carcinomas known to produce CEA.

The Two Hybridoma Antibodies Recognize Different Antigenic Determinants of CEA. In order to determine whether the antibodies from the two clones were reacting with identical or different antigenic determinants on the CEA molecule, [^{3}H]leucine-labeled antibodies from the two clones were tested for their binding capacity to unlabeled CEA adsorbed to the wells of polyvinyl plates in the presence of an

Table 1. Isotype analysis of antibodies from clones VII-23e and VII-37a*

Sample	Binding by isotype antibodies, cpm†			
	IgM	IgG_1	IgG_2	IgA
VII-23e	137	3665	284	95
VII-37a	134	410	2619	93
NMS‡	136	266	139	124

* CEA was adsorbed to the wells of polyvinyl plates (15) (Cooke, Alexandria, VA) by incubation of 0.1 ml of phosphate-buffered saline containing 10 μg of CEA for 2 hr at room temperature followed by saturation of the wells with 200 μl of a 1% bovine serum albumin solution in phosphate-buffered saline. The following reagents were then incubated sequentially in the wells for 2 hr at room temperature: culture fluids from clones VII-23e or VII-37a (100 μl undiluted) or normal mouse serum (100 μl diluted 1:20), goat antiserum specific for mouse IgM, IgG_1, IgG_2, or IgA (Meloy, Springfield, VA) (100 μl diluted 1:5000), immunoadsorbent-purified ^{125}I-labeled rabbit antibodies against goat IgG (100 μl containing 5 ng of antibodies representing 25,000 cpm).

† ^{125}I-Labeled rabbit anti-goat IgG antibodies bound in wells containing goat antisera to the indicated isotype.

‡ Normal mouse serum diluted 1:20 in phosphate-buffered saline.

excess of unlabeled antibody from each of the two clones. Fig. 2 shows that the binding of labeled antibody from clone 23 was markedly inhibited by unlabeled antibody from the same clone but not by antibody from clone 37. Similarly, the binding of labeled antibody from clone 37 was inhibited by antibody from the same clone but not by antibody from clone 23. These results clearly indicate that the antibodies from the two clones react with different antigenic determinants present on the CEA molecule.

Affinity Constant of the Two Monoclonal Antibodies. The affinity constant of the antibodies from the two clones was determined by measuring the binding of increasing amounts of ^{125}I-labeled CEA to a limited constant amount of antibodies. After 16-hr incubation at 37° C, CEA bound to antibodies was precipitated by ammonium sulfate. Fig. 3*A* shows the saturation curves obtained with antibodies from both clones. Fig. 3*B* shows the transformation of the same data into double reciprocal plots. The plots obtained for the two clones are linear, as expected for homogeneous antibodies. These results allow the calculation of the affinity (K_a) by determining the intercept of the negative extension of the abscissa (14, 16). The calculated affinity was 1.4×10^8 M^{-1} for clone 23 and 1.1×10^7 M^{-1} for clone 37. This difference in affinity may explain the difference of sensitivity of the radioimmunoassays developed with antibodies from the two clones.

Unlimited amounts of monoclonal anti-CEA antibodies such as those described herein can be produced, and they will be useful as standard reagents for the identification of CEA and for the characterization of the different antigenic determinants of this tumor marker.

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